

# Switching the mode of metabolism in the yeast *Saccharomyces cerevisiae*

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**The biochemistry of most metabolic pathways is conserved from bacteria to humans, although the control mechanisms are adapted to the needs of each cell type. Oxygen depletion commonly controls the switch from respiration to fermentation. However, *Saccharomyces cerevisiae* also controls that switch in response to the external glucose level. We have generated an *S. cerevisiae* strain in which glucose uptake is dependent on a chimeric hexose transporter mediating reduced sugar uptake. This strain shows a fully respiratory metabolism also at high glucose levels as seen for aerobic organisms, and switches to fermentation only when oxygen is lacking. These observations illustrate that manipulating a single step can alter the mode of metabolism. The novel yeast strain is an excellent tool to study the mechanisms underlying glucose-induced signal transduction.**  
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## INTRODUCTION

The glycolytic pathway and its individual enzymes are conserved during evolution, although mechanisms controlling carbon and energy metabolism have adapted to the needs of each species or cell type. Aerobic organisms respire pyruvate completely to CO<sub>2</sub> with oxygen (O<sub>2</sub>) as the terminal electron acceptor, thereby making maximal use of energy transformations for ATP produc-

tion. However, facultative aerobic organisms may add fermentation for fast energy production. For instance, glucose is fermented to lactate by human muscle cells. Similarly, the yeast *Saccharomyces cerevisiae* switches to a mixed respiro-fermentative metabolism, resulting in ethanol production, as soon as the external glucose concentration exceeds 0.8 mM (Verduyn *et al*, 1984). Hence, *S. cerevisiae* controls fermentation versus respiration primarily in response to the sugar level.

Aerobic ethanol production by *S. cerevisiae* is thought to depend on the relative capacities of the fermentative and respiratory pathways: high glucose levels result in a glycolytic rate exceeding that of the pyruvate dehydrogenase (Pdh) reaction, thereby generating an overflow towards pyruvate decarboxylase (Pdc) and hence ethanol production. At low external glucose levels and in the presence of oxygen, *S. cerevisiae* does not produce ethanol (Kappeli, 1986). Recent studies on human Pdh suggest that lactate accumulation in muscle cells is also due to an overflow of pyruvate towards lactate formation and is not a result of oxygen limitation in the mitochondria (Heigenhauser & Parolin, 1999; Parolin *et al*, 2000a,b). Such overflow occurs during exercise, when glycogen is broken down to ensure rapid ATP production (Hargreaves, 2000).

The uptake of glucose into *S. cerevisiae* is controlled by multiple hexose transporters (Hxts) (Ozcan & Johnston, 1999), which have different substrate specificity and affinity, and are expressed under different, overlapping conditions (Reifenberger *et al*, 1997). The availability of a yeast strain that lacks all those Hxts (Wieczorke *et al*, 1999) and that does not take up glucose opens the possibility to study the role of uptake in glycolytic metabolism and glucose-induced signalling. We reasoned that chimeric sugar transporters composed of parts of the low-affinity Hxt1 and the high-affinity Hxt7 could result in transporters with novel properties. One of the chimaeras, when expressed as the only glucose transporter, conferred a respiratory metabolism even at high external glucose concentrations. At the same time, the strain maintained its ability to ferment under anaerobic conditions. The data presented here show that manipulating one

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individual step, glucose uptake, can alter the mode of metabolic control. The characterization of this yeast strain provides a novel insight into the control of metabolism and signalling.

## RESULTS AND DISCUSSION

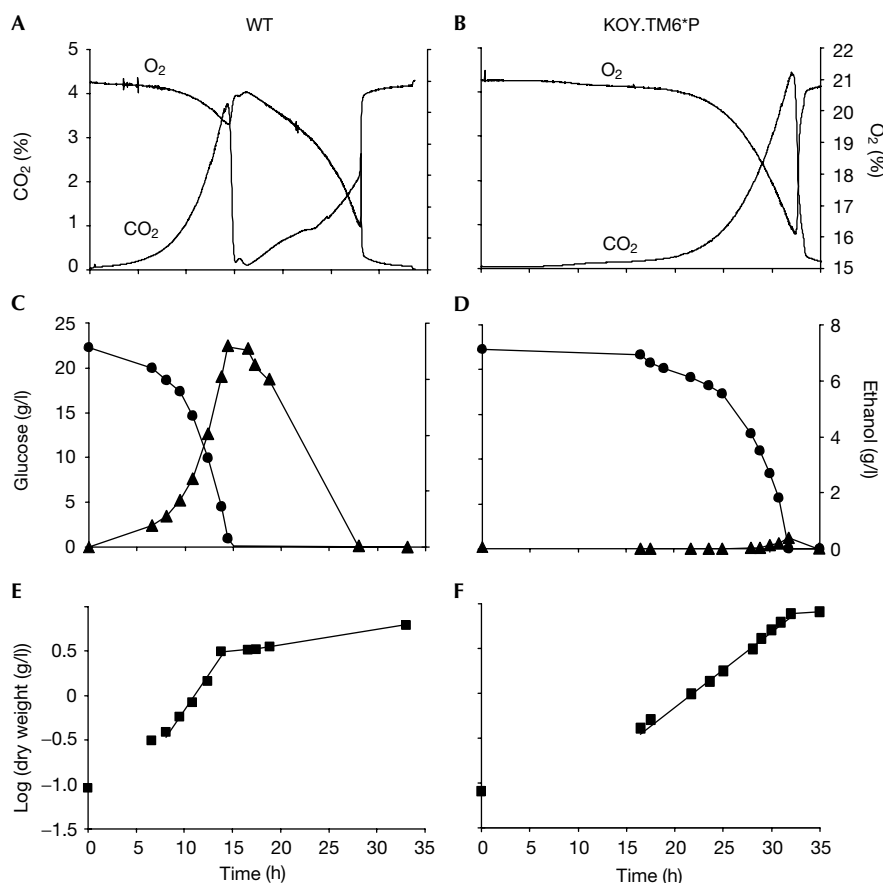
### An *S. cerevisiae* strain with a fully respiratory growth

During aerobic growth on fermentable carbon sources, *S. cerevisiae* controls fermentation versus respiration in response to the carbon source level or quality. Maximal ATP yield does not seem to be an issue for *S. cerevisiae* in its natural environment, and it has been interpreted that ethanol production instead provides a competitive advantage.

In this study, we are presenting an *S. cerevisiae* strain that switches to fermentation only when oxygen is removed. The strain we generated relies for sugar uptake on a chimeric sugar transporter composed of the low-affinity (100 mM) and the high-affinity (1–2 mM) transporters Hxt1 and Hxt7. They are 72% identical and are predicted to consist of 12 membrane-spanning domains (TMDs) (Ozcan & Johnston, 1999). The construct was integrated into the genome of the *hxt* null strain KOY.VW100 behind the truncated *HXT7* promoter (Hauf et al, 2000). A fusion in TMD6 (*TM6*) generated a nonfunctional glucose transporter. A serendipitous mutation introduced by PCR (*TM6\**, S279Y) generated a transporter that mediated growth of strain KOY.*TM6\**P (from here onwards called the *TM6\** strain) on

glucose. Hence, single point mutations can render a nonfunctional chimaera functional, and this approach could be instrumental in future structure–function analysis.

The wild type showed a carbon dioxide production and oxygen consumption profile (Fig 1A) typical for aerobic diauxic growth by *S. cerevisiae* (Fiechter et al, 1981; Diderich et al, 2001). Glucose was first catabolized fermentatively to carbon dioxide and ethanol plus minor amounts of other by-products (e.g. glycerol and acetate). A fraction of the sugar was catabolized respiratorily or used for biomass production. In the following phase, ethanol was respired to carbon dioxide and water, and partly incorporated into biomass (Fig 1C,E). The wild type had a respiratory quotient (RQ) of 3.4 (Table 1). In contrast, the *TM6\** strain exhibited a single growth phase (Fig 1B). The RQ of this strain was 1.0 (Table 1), consistent with an exclusively respiratory metabolism (Fig 1D). The strain relying on *Tm6\** for glucose transport produced only negligible amounts of ethanol (8 mM) and other by-products during the entire growth phase. Consequently, the *TM6\** strain attained a higher overall biomass yield (Fig 1E,F). Similar results were obtained at even higher glucose levels (5%, data not shown). Although the glucose consumption rate, that is, glycolytic rate, of *TM6\** was only about 20% of that of the wild type (Table 1), the specific growth rate was as high as 70% (Table 2). The wild type exhibited low and the *TM6\** strain high glucose affinity of the sugar uptake (Table 2).  $V_{\max}$  was only about 10% of that of the



**Fig 1** | Glucose, ethanol, dry weight and gas measurements during aerobic batch cultures of the wild type (WT) and *TM6\** in 2% glucose. (A,B) Oxygen consumption and carbon dioxide evolution. (C,D) Glucose (filled circle) and ethanol (filled triangles). (E,F) Dry weight (filled squares).

**Table 1** | Metabolic characteristics of the wild type and the *TM6\** strain in aerobic batch with 2% glucose and after shift to anaerobicity

Strain	Aerobically			Anaerobically		
	Glucose consumption rate (mmol/g h)	Ethanol production rate (mmol/g h)	RQ	Glucose consumption rate (mmol/g h)	Ethanol production rate (mmol/g h)	RQ
Wild type	16 ± 2	20 ± 3	3.4	20 ± 1	39 ± 7	∞
<i>TM6*</i>	3.5 ± 0.5	0.0	1.0	2.8–1.0	5.0–1.0	∞

Values are mean ± s.d. g, dry biomass; RQ, respiratory quotient.

**Table 2** | Kinetic data ( $V_{\max}$  and  $K_m$ ) of glucose transport, specific growth rates ( $\mu$ ) and oxygen consumption rates of the wild type and the *TM6\** strain

Strain	$V_{\max}$ (nmol/(mg protein)min)	$V_{\max}$ (%)	$K_m$ (mM)	$\mu$ (h <sup>-1</sup> )	$\mu$ (%)	Oxygen consumption rate (mmol/(g biomass)h)
Wild type	663 ± 31	100	76 ± 9.2	0.35	100	1.4 ± 0.3
<i>TM6*</i>	61 ± 2.1	9	3.5 ± 0.5	0.23	66	6.3 ± 0.4

Values are mean ± s.d.

**Table 3** | Specific activity (mU/mg protein) of invertase and Pdc for the wild type and the *TM6\** strain

Strain	Carbon source	Invertase	Pdc
Wild type	Ethanol	680 ± 100	100 ± 10
	Glucose	330 ± 10	1,470 ± 190
<i>TM6*</i>	Ethanol	620 ± 100	120 ± 1
	Glucose	4,240 ± 700	430 ± 12

Values are mean ± s.d. Pdc, pyruvate decarboxylase.

wild type (Table 2), consistent with glucose consumption data (Table 1).

Following the concept that alcoholic fermentation occurs once the glycolytic rate exceeds the capacity of the Pdh reaction, it appears that the low sugar uptake in *TM6\** keeps the pyruvate formation rate below the critical value (Kappeli, 1986). In addition, Pdc expression is stimulated by glucose to a lower degree in the *TM6\** strain (Table 3 and Fig 3). The respiratory rate is 4.5 times higher in the *TM6\** strain than in the wild type (Table 2).

**Fermentative growth under anaerobic conditions**

When shifted to anaerobic conditions, both the wild type and the *TM6\** strain show a transient drop in gas production (Fig 2). The *TM6\** strain continues to consume glucose and switches to fermentative metabolism, albeit at a much slower rate than the wild type (Fig 2 and Table 1). The increase in biomass (120%) observed for the *TM6\** strain was accompanied by only a 30% increase in cell number and a 75% decrease in budding index. Hence cells had ceased dividing and uncoupled glycolytic rate and growth.

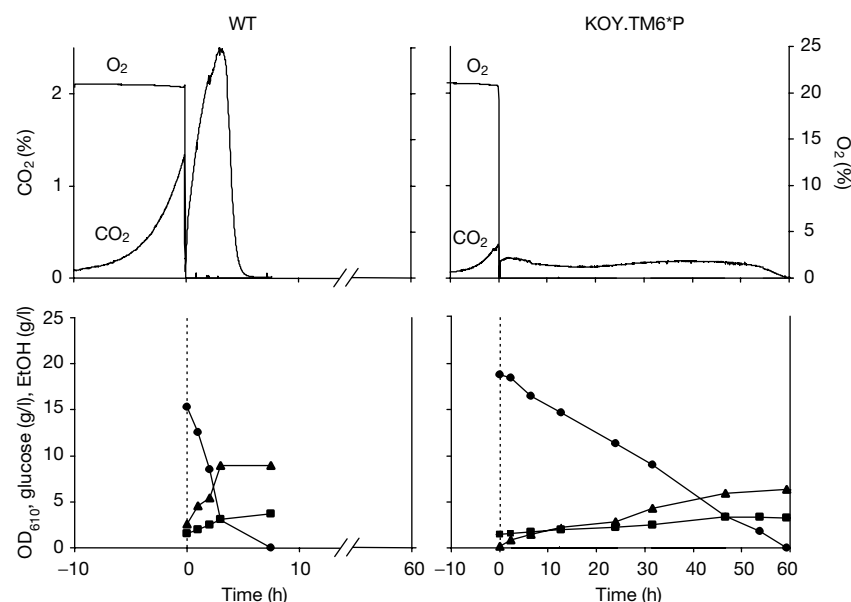
The basis for aerobic fermentation by yeast has been the subject of many studies. Our data show that altering one single metabolic step, glucose uptake, is enough to switch the mode of metabolism from fermentation to respiration. The behaviour of the

*TM6\** strain furthermore suggests that during evolution yeast has precipitously increased the capacity of glucose uptake and the glycolytic pathway, whereas that for the tricarboxylic acid cycle has not followed. The driving force for that evolution may have been a competitive advantage in the yeast's natural environment. It should be noted that the observed amplification of hexose transporter genes does not seem to contribute much to the increased uptake capacity, as single Hxts can sustain almost wild-type uptake capacity (Reifenberger *et al*, 1997). Rather, the differently expressed transporters allow the cell to adjust sugar affinity to substrate availability.

**Glucose-induced gene expression**

The presence of glucose, the preferred carbon and energy source, mediates a large number of changes in yeast and all other organisms investigated so far. A major issue in this regard is whether sensing occurs at the cell surface by membrane-localized receptors or internally; that is, at the level of metabolism. Both modes of sensing seem to exist in yeast (Rolland *et al*, 2002). To exemplify the usefulness of the *TM6\** strain to study glucose-induced regulatory effects, we have looked at two different phenomena, glucose repression and glucose induction of gene expression during derepressed (ethanol growth) and repressed (glucose growth) conditions. Different regulatory pathways mediate glucose repression itself. Repression of the *SUC2* gene at high glucose levels is controlled by the Snf1–Mig1/Mig2 system (Gancedo, 1998), whereas low glucose levels stimulate *SUC2* expression (Ozcan *et al*, 1997). In the *TM6\** strain, expression of *SUC2* is induced about tenfold by glucose levels that mediate repression in the wild type (Fig 3). This observation suggests that the *TM6\** strain perceives 2% glucose as less than about 0.2% glucose. Consistent with previous evidence (Gancedo, 1998), this is only possible if sensing occurs after the glucose uptake step at the metabolic level by the Snf1–Mig1 system.

Expression of the gluconeogenic genes *FBP1* and *MDH2* is controlled by the Snf1–Mig1 system and an induction mechanism involving the Cat8 and Sip4 regulators (Schuller, 2003). Significant expression of both genes was detectable in the *TM6\** strain on



**Fig 2** | The wild type (WT) and *TM6\** were grown aerobically to an OD<sub>610</sub> of 1–1.5 before shift to anaerobicity at time zero. Glucose (filled circles) and ethanol (EtOH; filled triangles) concentrations and optical density (filled squares) were followed. Oxygen consumption and carbon dioxide evolution were followed.

glucose (Fig 3), consistent with sensing inside the cell and with the notion that this strain perceives only a very low glucose level. Expression of *ADH2*, which is dependent on Snf1 for derepression and activated by Cat8 and Adr1 (Schuller, 2003), is completely derepressed in *TM6\** during glucose growth. Surprisingly, on ethanol, absolute expression levels of *FBP1*, *MDH2* and *ADH2* are higher in the *TM6\** strain than in the wild type (Fig 3). Perhaps the *Tm6\*p* hexose transporter causes a different efflux of glucose produced in gluconeogenesis and/or trehalose turnover, resulting in various metabolic pool sizes.

Derepression of *CYC1*, which encodes iso-1-cytochrome C in respiration, is Snf1 dependent (Wright & Poyton, 1990) and expression is induced by the activator Hap1 (Pfeifer et al, 1987). Glucose repression of *CYC1* expression is largely abolished in the *TM6\** strain (Fig 3), suggesting that also the Hap1 system senses carbon source availability internally rather than via membrane receptors.

The expression of genes encoding glycolytic and fermentative enzymes such as *PGK1*, *TPI1*, *PDC1* and *ADH1* is induced to a different degree by glucose (Fig 3). Their expression requires the regulator complex Rap1–Gcr1–Gcr2 (Chambers et al, 1995). The extent of induction depends on the glucose level and probably on glucose being taken up and metabolized (Muller et al, 1995). Our observations in the *TM6\** strain confirm this notion, as expression of these genes on glucose medium is diminished to different degrees.

Taking these results together, the *TM6\** and other similar strains (data not shown) are most useful tools to study glucose-induced regulatory effects and they will prove highly instrumental in further analyses.

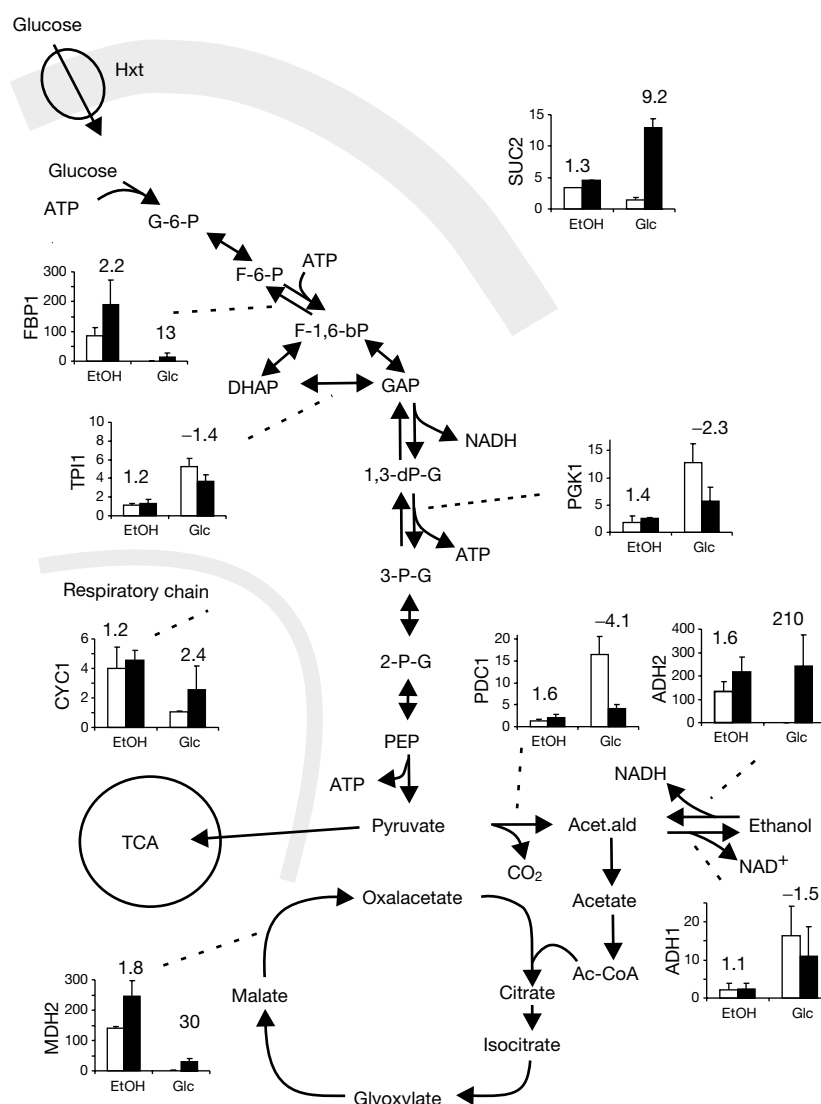
## Metabolic engineering

Over the last two decades, numerous attempts have been reported to redirect the flux of glucose towards respiration and to avoid

ethanol production at high extracellular glucose concentrations. Eliminating the pathway from pyruvate to ethanol has failed, not least because Pdc is necessary for the production of cytosolic acetyl-CoA (Flikweert et al, 1996). A mutant devoid of the four most important alcohol dehydrogenases still produced ethanol (Drewke et al, 1990). It has also been attempted to increase respiratory activity by alleviating glucose repression. This resulted in a shift towards fermentation initiated at higher glucose levels and a relief from glucose repression but only a partial redirection of metabolism to respiration (Diderich et al, 2001). A similar result was obtained by different expression levels of the high-affinity transporter Hxt7 (Ye et al, 1999). The availability of the *TM6\** strain hence constitutes a breakthrough because it demonstrates that the mode of metabolism can be completely altered by engineering a single metabolic step—uptake. It provides research with a novel unique tool to study metabolic control in the yeast model system. This strain offers the possibility to study glucose signalling also at high extracellular glucose concentrations and maintaining respiratory growth. It also provides industry with a fully respiratory strain, which gives a higher biomass yield and potentially increased heterologous protein production in simple batch cultures.

## METHODS

**Yeast strains.** All strains were derived from CEN.PK2-1C (*MATa*, *leu2-3*, *112 ura3-52 trp1-289 his3-Δ MAL2-8<sup>c</sup>SUC2 hxt17Δ*) (van Dijken et al, 2000) in which the auxotrophic markers *HIS3*, *TRP1*, *LEU2* and *URA3* had been reintroduced. In EBY.VW4002, all relevant hexose transporters have been deleted (Wieczorke et al, 1999) and the markers *HIS3*, *TRP1* and *LEU2* have been reintroduced resulting in the *hxt* null strain KOY.VW100. In EBY.VW4002, the *HXT3-HXT6-HXT7* locus has been replaced by a cassette comprising the truncated *HXT7* promoter (Hauf et al, 2000) followed by the *KIURA3* gene and the *HXT7* terminator.



**Fig 3** | Relative expression levels in the wild type (open bars) and *TM6\** (closed bars) under derepressed (ethanol growth) and repressed (glucose growth) conditions. Numbers: fold differences in expression in *TM6\** compared with the wild type either at ethanol (EtOH) or glucose growth. Bars indicate mean  $\pm$  s.d. SUC2, invertase; FBPI, fructose 1,6 bisphosphatase; TPI1, triose-phosphate isomerase; PGK1, phosphoglycerate kinase; PDC1, pyruvate decarboxylase1; ADH1/2, alcohol dehydrogenase 1/2; CYC1, iso-1-cytochrome C; MDH2, malate dehydrogenase 2; TCA, tricarboxylic acid cycle.

**Construction of *TM6\** strain.** *TM6* was generated using overlap extension PCR (Ho *et al*, 1989). The *HXT1* (bp 1–741) portion was amplified from the plasmid pHXT1-2 (Reifenberger *et al*, 1997) and the *HXT7* (bp 742–1713) portion from p21 (Reifenberger *et al*, 1995). The construct was integrated by recombination into KOY.VW100, replacing the *KIURA3* marker. Transformants were selected first on 2% YPD plates and then on 1% maltose, 5-fluoroorotic acid YNB plates. Sequencing of *TM6* revealed a point mutation (S279Y, *TM6\**). The strain was finally made prototrophic by integration of the *URA3* marker. The *TM6\** construct was amplified from the *TM6\** strain and re-transformed into a new KOY.VW100. The same phenotype was obtained.

**Medium and cultivation in fermentors.** Bioreactor fermentations were performed in 1.5 l of 2  $\times$  concentrated complete minimal medium (Verduyn *et al*, 1992), 2% glucose and polypropylene

glycol P2000 as anti-foam at 30  $^{\circ}$ C, 1,500 r.p.m., pH 5.0, and an air flow of 0.75 l/min. Carbon dioxide production and oxygen consumption were measured on-line (type 1308, Bruel and Kjaer). The RQ was calculated as the ratio of the carbon dioxide production rate to the oxygen consumption rate.

**Oxygen consumption rate.** The oxygen consumption rate was measured in a Cyclobios oxygraph (A. Paar, Austria) during logarithmic growth on glucose by transferring 2.2 ml of cells directly into the oxygraph.

**Anaerobic shift.** Cultures were grown as described above until an OD<sub>610</sub> of 1–1.5. The culture was shifted to anaerobicity (N<sub>2</sub> gas) and Ergosterol/Tween 80 (10  $\mu$ g/l/0.42  $\mu$ g/l) was added.

**Biochemical determinations.** For dry weight, 5 ml of the culture was centrifuged, washed twice in MilliQ water and dried for 24 h at 110  $^{\circ}$ C. The glucose, ethanol, glycerol and acetate



concentrations in the medium supernatant were determined using enzymatic combination kits.

**Sugar uptake analysis.** Sugar uptake of  $^{14}\text{C}$ -glucose was assayed on logarithmic cells at a cell density of 7.5% w/v (Walsh *et al*, 1994). Protein was determined (Lowry *et al*, 1951) with bovine serum albumin (BSA) as a standard. Data from at least two independent experiments (performed in duplicate) were analysed using computer-assisted nonlinear regression. The calculations indicated one-component Michaelis–Menten uptake kinetics.

**Activity measurements.** Pdc activity (Schmitt *et al*, 1983) and invertase activity (Goldstein & Lampen, 1975) were measured according to established protocols. Protein was measured using the Bio-Rad DC protein assay with BSA as a standard.

**mRNA and quantitative real-time PCR.** Cells were cultivated in fermentors with 1% ethanol and 5 × concentrated minimal medium (Verduyn *et al*, 1992) and pulsed with 5% glucose at an OD<sub>610</sub> of 1–1.5. Samples were taken during ethanol growth and at glucose concentrations between 15 and 25 g/l, mRNA was extracted, DNase treated and controlled on agarose gel. Reverse transcription (Superscript II, Invitrogen) using pd(T)<sub>12–18</sub> as primers and quantitative real-time PCR assays were performed in an iCycler (Bio-Rad). PCR products were checked by agarose gel and melting curve analysis. Expression data were normalized against *IPP1* and *ACT1*. Experiments were repeated at least twice and verified by northern blot analysis.

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